MOLECULAR AND CELLULAR BIOLOGY, Feb. 1985, p. 410–413
0270-7306/85/020410-04$02.00/0

Vol. 5, No. 2

A Vector That Replicates as a Plasmid and Can Be Efficiently Selected in B-Lymphoblasts Transformed by Epstein-Barr Virus

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Received 20 September 1984/Accepted 30 October 1984

Epstein-Barr virus (EBV) transforms human B-lymphocytes into proliferating blasts which are efficiently established into cell lines. The viral DNA in these cell lines is usually present as complete, unintegrated plasmid molecules. A *cis*-acting element of EBV, *oriP*, permits plasmid maintenance in adherent cells that carry EBV DNA. We constructed a vector, pHEBo, that carries *oriP* and showed that it is also efficiently maintained as a plasmid when introduced into EBV-transformed B-lymphoblasts. The pHEBo vector carries the coding sequences for the *hph* gene from *Escherichia coli* such that it can be expressed in mammalian cells and confers resistance to the antibiotic hygromycin B. Hygromycin B kills EBV-transformed lymphoblasts at concentrations of 50 to 300 μg/ml. The combination of *oriP* plus the expressed *hph* gene makes pHEBo useful for the stable introduction of genes on plasmids into EBV-transformed lymphoblasts. Because pHEBo is derived from the plasmid pBR322 it can be easily isolated from lymphoblasts by reintroduction into *E. coli*.

Epstein-Barr virus (EBV) readily transforms human B-lymphocytes into established, proliferating cell lines (16, 18). In fact, transformation by EBV is the usual mode of establishing cell lines from both normal individuals and those who carry identified germ-line mutations. The EBV-transformed lymphoblast is usually euploid, secretes immunoglobulin, and carries multiple, complete copies of viral DNA as plasmid molecules (16, 18). To study transformation by EBV, we sought methods to introduce DNAs into the transformed cells such that the DNAs would be maintained and expressed. We constructed a vector, pHEBo, that contains the *oriP* element of EBV and the *hph* gene from *Escherichia coli*. It can be efficiently selected and is maintained as a plasmid in EBV-transformed lymphoblasts. The *oriP* element acts in *cis* and permits plasmid replication in cells that carry EBV DNA, as shown previously with adherent, somatic cell hybrids (17). Gritz and Davies showed that the *hph* gene confers resistance to hygromycin B in yeast (3), and we show here that it also confers a similar resistance in mammalian cells. We found that the pHEBo vector can be readily introduced into EBV-transformed lymphoblasts by electroporation or protoplast fusion. After its introduction into EBV-transformed lymphoblasts, the pHEBo vector can yield up to 10^5 more resistant colonies per μg of DNA than does a related vector lacking *oriP*. Among the cells surviving the attempt to introduce DNA, up to 3% also survive selection to grow as resistant colonies, having 1 to 60 copies of pHEBo per cell but usually having an average of 10 plasmid molecules per cell. This vector should prove useful in introducing DNAs as plasmids into EBV-transformed cells to study the virus, to study the varied phenotypes of these established and transfected cell lines, and to isolate genes from recombinant DNA libraries which can be expressed from this plasmid.

Available methods for the dominant selection of DNAs in EBV-transformed B-lymphoblasts were unsatisfactory. Cells selected to be resistant to mycophenolic acid proliferate poorly, and the derivative of neomycin, G418, is often toxic to lymphoblasts only at high concentrations (unpublished observations). We therefore attempted to apply to mammalian cells the selection for resistance to the aminoglycoside hygromycin B. We first compared the toxicities of G418 and hygromycin B to B-lymphoblastoid cell lines and found hygromycin B to be 3 to 10 times more toxic on a weight basis to these cells (Table 1). We then constructed the vector pHyg to express the *hph* gene (an *E. coli* gene encoding a hygromycin B phosphotransferase) via the transcriptional promoter and polyadenylation signals of the thymidine kinase gene (*Tk*) of herpes simplex virus type 1 (Fig. 1). To permit the vector to replicate as a plasmid in EBV-transformed cells, a fragment of EBV DNA containing *oriP* was added to pHyg to generate pHEBo (Fig. 1).

The two plasmids, pHyg and pHEBo, were efficiently introduced into EBV-transformed lymphoblasts by protoplast fusion (12, 13) or electroporation (10), and survivors of exposure to hygromycin B were selected (Table 2). For protoplast fusion *E. coli* DH1 cells (optical density at 650 nm, 0.6) carrying the desired plasmid were grown overnight in the presence of chloramphenicol (200 μg/ml), converted to protoplasts, and mixed at a ratio of 1,000:1 with mammalian cells that had first been washed three times in Ca^{2+}-free minimal essential medium. The mixed cells were spun into a pellet, gradually suspended in 1 ml of 50% polyethylene glycol (average molecular weight, 1,500) in Ca^{2+}-free minimal essential medium for 1 min at 37°C, slowly mixed for 1 min, diluted to 20 ml with Ca^{2+}-free minimal essential medium during 4 min, pelleted, and incubated in RPMI 1640 medium supplemented with serum, streptomycin (100 μ/ml), and gentamycin (50 μg/ml). One day later, between 1 and 20% of the mammalian cells were found to have survived the fusion process as determined by their excluding eosin yellow dye. At 24 to 48 h after the fusion, the cells were distributed into 24-well plates at concentrations ranging from 30 to 3×10^5 cells per well in medium containing 300 μg of hygromycin per ml. The second method we used to introduce DNAs into lymphoblasts was electroporation (10) as developed by Huntington Potter (10a). Cells to be electroporated were suspended at 0°C in phosphate-buffered saline (0.5×10^7 to 2×10^7 cells in 0.5 ml) in a plastic cuvette containing 1-cm-wide electrodes separated by 1 cm. To the cells was added 5 to 10 μg of plasmid DNA. One electrode was then attached to an Isco 494 power source set at 2,000 V and at that minimal

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TABLE 1. Growth of cell lines in the presence of various concentrations of G418 and hygromycin B[a]

Cell line (reference)	Growth in the presence of:											
	G418 (μg/ml)[b]						Hygromycin B (μg/ml)[c]					
	0	300	600	900	1,200	1,500	0	50	100	200	300	400
721 (6)	+	+	+	+	+/−	−	+	+	−	−	−	−
Raji (11)	+	+	+	+	+/−	−	+	+	−	−	−	−
Daudi (7)	+	+	+	+	+	+/−	+	+	+	−	−	−
GG68 (4)	+	+	+	+	+/−	−	+	+	+	+	+/−	−
WIL2 $TK^{-/-}$ (14)	+	+	+	+/−	−	−	+	+	+	−	−	−
B95-8 (9)	+	+	+	+	−	−	+	−	−	−	−	−

[a] Lymphoblastoid cells were plated at 2×10^4 per ml in RPMI 1640–10% fetal bovine serum for the human cells and RPMI 1640–5% calf serum for the B95-8 cells in the various concentrations of G418 or hygromycin B, and survival was followed for 10 days. Symbols: +, an increase in cell number; +/−, a decrease in cell number but with some viable cells on day 10; and −, no surviving cells on day 10.

[b] G418 was purchased from GIBCO Laboratories and made as a stock solution at 100 mg/ml in phosphate-buffered saline (the stated purity of the drug 46%, was not considered in making these solutions).

[c] Hygromycin B, purchased from Calbiochem, was made as a stock solution at 10 mg/ml in phosphate-buffered saline and brought to 3.5×10^{-2} M with glacial acetic acid to neutralize the solution. We found that the biological half-life of solutions of some samples of hygromycin B at 4°C is on the order of 10 days, indicating that their shelf-life as a solution can be limited.

current which just permitted voltage regulation. A transient current was then passed through the cuvette by bringing a lead from the power source close enough to the second electrode to permit a discharge. We measured this discharge to result in a transient current of 3 to 6 amps with a rise time of less than 1 μs and a time constant for decay of approximately 30 ms. The cells were removed from the cuvette and diluted into 10 ml of RPMI 1640 plus serum. One day later, between 1 and 40% of the cells were measured by dye exclusion to have survived electroporation. At 24 to 48 h after electroporation, the cells were diluted into selective medium as described above for protoplast fusion.

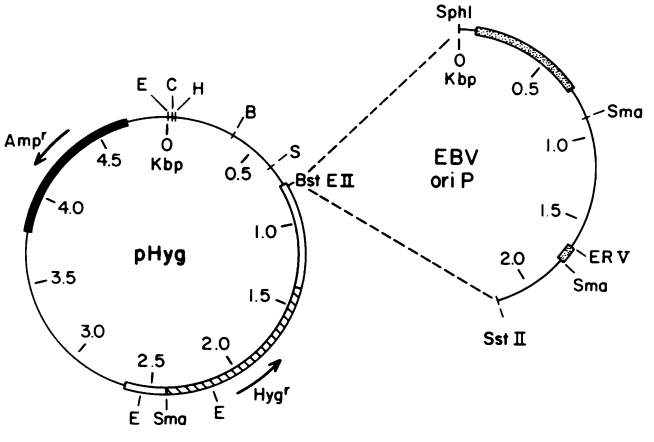

FIG. 1. The construction of pHEBo from pHyg and *oriP*. The plasmid pHyg, which confers resistance to hygromycin B by transcriptional controls from the herpes simplex virus type 1 *tk* gene was constructed from pKan2 (14) and pLG89 (3). pKan2 was digested with *Sma*I and *Bgl*II to remove the sequences derived from Tn*5*. pLG89 was digested with *Bam*HI to obtain the *hph* gene which had been inserted into pLG89 with *Bam*HI linkers. The *hph* gene was inserted into the digested pKan2 by using blunt-end ligation at the *Sma*I site and cohesive-end ligation at the *Bgl*II site. The *Sma*I and *Bgl*II sites of pKan2 were lost during ligation. The plasmid pHEBo is the plasmid pHyg carrying the EBV *oriP*. The EBV DNA that spans 7,333 to 9,516 base pairs on the B95-8 map (1) and contains *oriP* was excised from plasmid pΔSstII-3 (17) by digestion with *Sph*I and *Sst*II and inserted into the *Bst*EII site of pHyg by blunt-end ligation. The *Bst*EII, *Sph*I, and *Sst*II sites were all lost in this process. Shown are the relevant endonuclease sites: E, *Eco*RI; C, *Cla*I; H, *Hin*dIII; ERV, *Eco*RV; B, *Bam*HI; S, *Sal*I; *Bst*EII; Sma, *Sma*I; *Sph*I; and *Sst*II. The thick line indicates the beta-lactamase gene of pBR322, the open boxes indicate the regions carrying the promoter and polyadenylate addition signals derived from the herpes simplex virus type 1 gene, the hatched box indicates the *hph* gene of *E. coli* which encodes resistance to hygromycin B, and the stippled boxes indicate two regions of EBV DNA which are both required for *oriP* function (unpublished observations).

To measure the efficiency with which the lymphoblasts assimilated DNA transiently, simian virus 40 DNA was introduced into Raji cells (11) by electroporation, and the cells were assayed for simian virus 40 T-antigen 36 h later by immunofluorescence. In this experiment 17% of the cells survived electroporation and 4 to 5% of the survivors expressed T-antigen, indicating that in this case minimally 4 to 5% of the survivors took up the added DNA.

Among the cells exposed to pHyg during electroporation, none became resistant to hygromycin B (Table 2), indicating that expression of pHyg was not maintained efficiently in these cells (no survivors from 1.5×10^6 viable cells selected). At least 2 to 3% of Raji or Daudi cells (7) and, on the other hand, 0.1% of WIL2 TK^- cells (14) exposed to pHEBo proliferated and were resistant to hygromycin B. These efficiencies are likely to be underestimations because they

TABLE 2. Selection of resistant cells after electroporation and protoplast fusion

Recipient cells	Method of introducing DNA[a]	DNA introduced	Proportion of cells resistant[b] to hygromycin B
Raji	Electroporation	pHyg	$<1/1.5 \times 10^6$
Daudi	Electroporation	pHyg	$<1/1.5 \times 10^6$
WIL2 TK^-	Electroporation	pHyg	$<1/10^6$
Raji	Protoplast fusion	pHEBo	$1/10^4$
Daudi	Protoplast fusion	pHEBo	$1/2 \times 10^4$
Raji	Electroporation	pHEBo	1/40
Daudi	Electroporation	pHEBo	1/30
WIL2 TK^-	Electroporation	pHEBo	$1/10^3$

[a] Survival of cells exposed to electroporation ranged from 3 to 20%, and survival after protoplast fusion ranged from 1 to 2%.

[b] At 48 h after attempting introduction of DNA, cells were counted for viability and plated in RPMI 1640–10% fetal bovine serum at 10-fold serial dilutions into 24-well dishes (6 wells per dilution) and selected with 300 μg of hygromycin B per ml. Shown is the proportion of resistant cells relative to the number of initially viable cells plated.

do not adjust for cloning efficiencies of the survivors which are less than one. The high efficiency of resistance to hygromycin B provided by pHEBo in Raji cells was close to the percentage of Raji cells that take up and express simian virus 40 DNA transiently. This similarity indicates that if pHEBo can be introduced into EBV-transformed cells, it can provide continued resistance to hygromycin B.

Yates et al. have shown that vectors that include *oriP* replicate as plasmids in somatic cell hybrids containing EBV DNA (17). We therefore determined whether pHEBo was present as a plasmid in hygromycin B-resistant Raji and Daudi cells. DNA of low molecular weight was isolated by the selective extraction method of Hirt (5) from 10 resistant

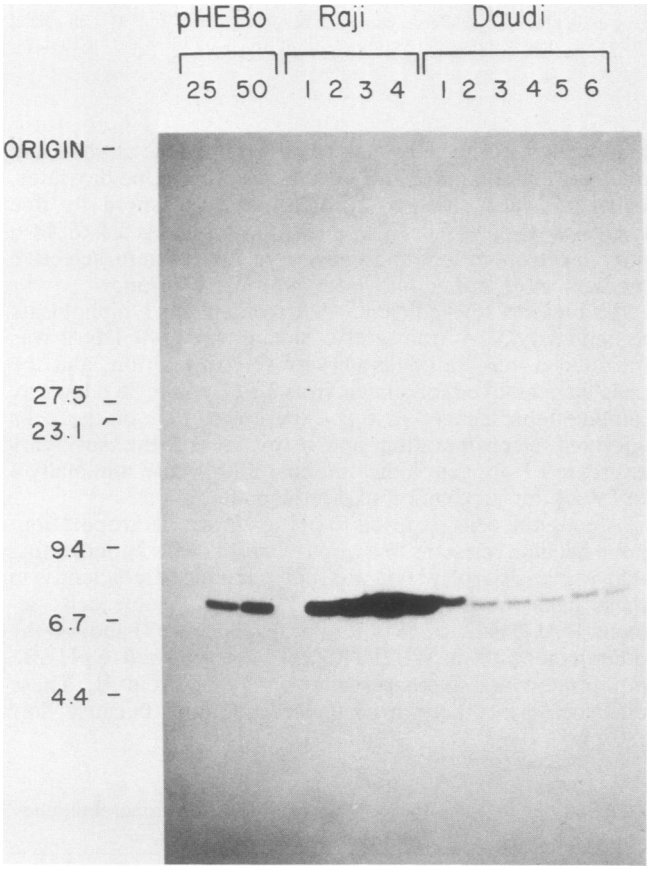

FIG. 2. DNAs isolated by Hirt extraction from 10^6 cells were digested with *Hin*dIII and separated by electrophoresis in a 0.5% agarose gel. On the same gel were run a *Hin*dIII digest of λ DNA to provide molecular weight markers, denoted as lengths in kilobase pairs, and a *Hin*dIII digest of pHEBo (25 and 50 pg) to aid in quantification. The DNAs were transferred to nitrocellulose (15) and detected by nucleic acid hybridization with ^{32}P-labeled pHyg as a probe, followed by autoradiography. The signals were quantified by excising the appropriate pieces of nitrocellulose and counting them in a scintillation counter. Raji 1 and 2 were derived after protoplast fusion from wells in which 7,500 cells were plated, and they contain 13 and 26 copies of plasmid, respectively; Raji 3 and 4 were derived after electroporation from wells in which 40 cells were plated, and they contain 60 and 28 copies, respectively; Daudi 1 was derived after protoplast fusion from a well in which 22,500 cells were plated, and it contains eight copies of plasmid; Daudi 2 through 6 were derived after electroporation from wells in which 3,000, 3,000, 30, 30, and 30 cells were plated, respectively, and each contains 1 to 2 copies of plasmid per cell. In quantifying the numbers of copies of plasmids per cell, one copy equalled 450 cpm.

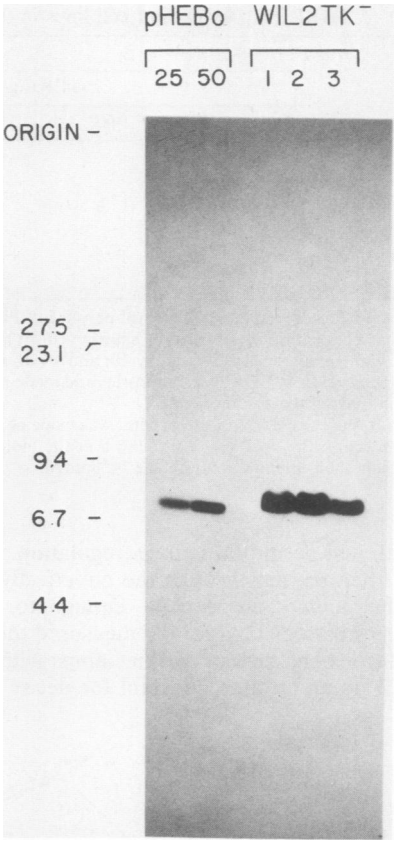

FIG. 3. Total cell DNAs were isolated from 10^6 cells, cleaved with *Hin*dIII, and analyzed as described in the legend to Fig. 2. WIL2 TK$^-$ 1 through 3 were derived after electroporation from wells in which 180, 1,800, and 1,800 cells were plated, respectively, and they contain 12, 14, and 9 copies of pHEBo per cell respectively. In the quantification experiment, one copy of the plasmid gave 530 cpm.

populations or clones. The isolated DNAs were analyzed by the method of Southern (15), and plasmids were detected by nucleic acid hybridization, using ^{32}P-labeled pHyg as a probe (Fig. 2). pHEBo was detected in the isolated DNAs at a frequency of 1 to 8 copies per Daudi cell and 13 to 60 copies per Raji cell (Fig. 2). The number of plasmid copies of pHEBo per cell appeared to be more related to the recipient cell than to the method of its introduction (Fig. 2).

We also analyzed DNAs from WIL2 TK$^-$ cells which were rendered resistant to hygromycin B after electroporation with pHEBo for the presence of integrated pHEBo DNA. Conceivably, pHEBo could integrate into either a human chromosome or an EBV plasmid residing in WIL2 TK$^-$ cells. Were homologous recombination between plasmid DNAs in lymphoblasts to be efficient, the pHEBo DNA sequences might be integrated into resident EBV plasmids at the site of *oriP*. The total cell DNAs of three presumptive clones isolated by dilution were digested with *Hin*dIII. *Hin*dIII cleaves EBV DNA, leaving *oriP* on a fragment of greater than 50 kilobase pairs, and it cleaves pHEBo once. In a Southern analysis the pHyg probe only detected a DNA the size of linearized pHEBo, indicating that in the three cell lines tested pHEBo was present only as an unintegrated plasmid DNA at 9 to 14 copies per cell (Fig. 3). A 96-fold-longer exposure than that shown in Fig. 3 could have detected 0.1 copy of pHEBo per cell, but no candidates for integrated molecules were observed (data not shown).

The combination of the *hph* gene of *E. coli* and the *oriP* element of EBV in a vector permits this vector to be selected efficiently in EBV-transformed lymphoblasts. We introduced pHEBo into all of the cell lines shown in Table 1 which included an in vitro transformant (721) as well as cell lines from a normal individual (WIL2 TK^-) and from three Burkitt lymphoma patients. In general, these cells do not take up DNA readily when the method of Graham and Van der Eb (2) is used (unpublished observations) but do so when either protoplast fusion or electroporation is used (Table 2). The vector pHEBo is present in these selected cells as a plasmid at anywhere from 1 to 60 copies per cell (Fig. 2), although the copy number is usually on the order of 10 per cell (unpublished observations). We have not yet found an example in which pHEBo is integrated in cells that contain EBV genomes (Fig. 3; unpublished observations). By using the method Yates et al. have described previously (17), pHEBo can be readily isolated from 10^6 hygromycin-resistant lymphoblasts and successfully shuttled back into *E. coli*.

One striking finding of these studies is the magnitude of the increased efficiency of pHEBo over pHyg in rendering cells resistant to selection after transfection. This increase arises from a route mechanistically different than similar increases caused by the inclusion of a viral transcriptional enhancer in a selectable vector. pHEBo and pHyg contain no known transcriptional enhancers. Transcriptional enhancers permit the expression of integrated copies of plasmids that would otherwise not be expressed (8). pHEBo is not found integrated into cell DNA in cells carrying EBV (Fig. 3). In cells lacking EBV DNA, pHEBo, like other selectable plasmids carrying *oriP*, does not replicate as a plasmid and yields no more colonies resistant to hygromycin B than does pHyg (17; J. Yates, N. Warren, and B. Sugden, Nature, in press). Together, these observations indicate that the efficient selection of pHEBo in EBV-transformed cells results at least in part from this vector being maintained as a plasmid in these cells.

The high efficiency with which pHEBo can be introduced, selected, and maintained as a plasmid in EBV-transformed cells indicates that it can provide an easy route to cloning a subset of eucaryotic genes. If the gene product is expressed when cloned and can be selected or screened for, then constructing a library of the DNA of a species as recombinant pHEBo clones, introducing these recombinants into an EBV-transformed lymphoblast, selecting the hygromycin B-resistant colonies, and selecting or screening among them for the desired gene product should yield either the gene itself or one that induces its expression. The pHEBo clone containing the gene of interest could then be readily transferred into *E. coli* for characterization (17).

We thank Linda Gritz and Julian Davies for providing us with their plasmid carrying the *hph* (hygromycin B resistance) gene before publication, Bart Barrell and co-workers for providing EBV DNA sequences before publication, William Summers and Michael Cole for introducing us to Huntington Potter's method of electroporation, and our colleagues at the McArdle Laboratory for critically reviewing the manuscript.

This work was supported by Public Health Service grants CA-22443, CA-97175, and 5 T32 CA-09075 from the National Institutes of Health. B.S. was supported by faculty research award 023 from the American Cancer Society.

ADDENDUM IN PROOF

Santerre et al. (**R. F. Santerre, N. E. Allen, J. N. Hobbs, Jr., R. N. Rao, and R. J. Schmidt,** Gene **30**:147–156, 1984) have used the *hph* gene from a different source in a different construction to confer resistance to hygromycin B in mouse L cells.

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